Research Article

In Vitro Assessment of Tick-Borne Encephalitis Vaccine: Suitable Human Cell Platforms and Potential Biomarkers

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Abstract

Tick-borne encephalitis (TBE) virus causes a severe disease that can lead to permanent neurological complications. The whole inactivated TBE vaccine is highly effective, as proven by high seroconversion rates and near eradication of the disease in countries where vaccination programs have been implemented. TBE vaccine potency testing currently requires the use of *in vivo* methods that present issues of reproducibility as well as animal discomfort. As an alternative, public and private entities are currently exploring a batch-to-batch consistency approach that would demonstrate conformity of a newly produced vaccine batch with a batch of proven *in vivo* efficacy with respect to a range of measurable *in vitro* quality parameters.

To identify a suitable cellular platform to be used in a panel of *in vitro* batch-to-batch assessments for the TBE vaccine, we exposed human cell-based systems, both of primary origin and cell line-derived, to vaccine formulations of high and low quality. Following stimulation, cell responses were evaluated by assessing the expression of selected genes by RT-qPCR. Our findings show that the expression of interferon-stimulated genes differed after treatment with non-adjuvanted vaccine batches of different quality in peripheral blood mononuclear cells (PBMCs) and in monocyte-derived dendritic cells, but not in monocyte-free PBMC suspensions nor in cell line-derived immune cells.

These results indicate suitable platforms and potential biomarkers for a cell-based assay that, together with other immunochemical analyses, could serve for batch-to-batch assessment of the TBE vaccine, reducing, and eventually replacing, *in vivo* methods for potency testing.

1 Introduction

Tick-borne encephalitis virus (TBEV) is an arthropod-borne flavivirus endemic in forested areas across Europe and Asia and the etiological agent of a neuroinvasive illness that can lead to severe long-term sequelae (Haglund and Günther, 2003; Dörrbecker et al., 2010). Approximately 13,000 TBEV-related cases of human encephalitis and meningitis are reported annually worldwide (Amicizia et al., 2013; Bogovic and Strle, 2015). The incidence of the disease has increased by more than 300% in Europe and Russia in the last 30 years because of climate and socio-economical changes (Süss, 2011; Nah et al., 2020). Existing TBE vaccines are highly immunogenic and provide at least 10 years of antibody persistence (Beran et al., 2018) through the induction of envelope protein-directed antibodies and of TBEV-specific CD4^{+} T cells (Kubinski et al., 2020). Endemic countries where vaccination measures have been implemented have seen a dramatic decline in the disease, with a rate of protection of over 95% (Heinz et al., 2007).

Currently, several licensed TBE vaccines are available across central Europe, Russia and Asia. All consist of formalin-inactivated whole virus formulations and differ in the viral strains and the excipients used (Lehrer and Holbrook, 2011). Before release,

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the European vaccines FSME-IMMUN (Pfizer) and Encepur (GlaxoSmithKline) must undergo mandatory batch validation by means of *in vivo* potency testing, as required by the European Pharmacopoeia (2008). Each newly produced batch is compared to a reference batch of proven quality by assessing the protection rate of immunized mice infected with a lethal viral dose. The procedure presents conspicuous disadvantages, from causing significant pain and distress to a large number of animals, to showing potency variations of up to 300% (Holzmann et al., 2011). Additionally, the phylogenetic distance between laboratory animals and humans may limit the predictive value of such *in vivo* tests (Leist and Hartung, 2013).

In the context of vaccine potency testing, a "consistency approach" has been proposed to reduce or replace the use of animal tests. This approach is based on a lot-release strategy in which *in vivo* tests may only be needed for the reference lots; *in vitro* assays can then be used to assess the conformity of new vaccine batches to earlier lots of proven safety and efficacy (De Mattia et al., 2011). Already, several *in vitro* techniques provide the opportunity of investigating the functional features of vaccines on models for innate or adaptive immune responses (Drake et al., 2012; Herrera-Rodriguez et al., 2019). Antigen-presenting cells (APCs), as the sentinels of the immune system and main targets and vectors of TBEV during primary viremia (Růžek et al., 2019), represent an ideal candidate platform for testing vaccine-induced innate immune responses. APCs and APC-like cell lines have been used regularly to analyze vaccine functions *in vitro* through multiparametric techniques (Vandebriel and Hoefnagel, 2012; Banchereau et al., 2014; Stoel et al., 2015) and have been shown to respond to vaccines in characteristic and vaccine-specific ways (Tapia-Calle et al., 2017).

In this study, we aimed to identify a cellular platform that could serve to reproducibly assess cellular responses induced by TBE vaccine batches. The cellular platform for such an assay should therefore satisfy two criteria: to display up- (or down-) regulation of biomarkers indicative of the vaccine quality – with distinctive responses to high-quality (conforming to product specification) and low-quality (non-conforming) vaccine batches – and to respond in a reproducible manner. We assessed a human cell line-based system, advantageous for relying on a consistent source of cells not subject to donor-dependent variation, and a primary cell-based system that preserves important *in vivo* properties (Ryu, 2017). The cell activation following treatment with TBE vaccine formulations was evaluated in THP-1-derived cells as candidates for cell line-based APC models (Berges et al., 2005; Bosshart and Heinzelmann, 2016) and in cryopreserved human peripheral blood mononuclear cells (PBMCs) and subpopulations derived from these cells.

THP-1-derived cells did not demonstrate TBE vaccine-specific activation and thus proved unsuitable for our purposes. In contrast, primary cell-based platforms responded specifically to the non-adjuvanted TBE vaccine through increased transcription of interferon-stimulated genes. Using selected interferon-stimulated genes (ISGs) as biomarkers in PBMCs, we were able to identify differential innate responses to conforming and non-conforming vaccine batches in an assay that is highly sensitive to differences in the product formulation. Our results indicate that a primary cell-based system can successfully be included in an array of *in vitro* methods for evaluation of the conformity of TBE vaccine batches.

2 Materials and methods

2.1 Vaccines and virus
Vaccine formulations
Non-adjuvanted TBE vaccine ("NAV"; 60 μg/mL protein) and the alum-adsorbed Encepur® vaccine ("vaccine"; 3 μg/mL protein, 2 mg/mL aluminum hydroxide) were kindly provided by GlaxoSmithKline (GSK, Marburg, Germany). NAV, the antigen-containing fraction of the Encepur® vaccine, consists of whole, formalin-inactivated TBEV in a 42% sucrose solution and thus contains virions including structural proteins and viral genome. Encepur is prepared from NAV by dilution of the inactivated virus and addition of the adjuvant (Holzmann et al., 2011). To produce non-conforming batches, NAV was heat-treated at 42°C for 4 weeks or at 100°C for 15 min in glass vials ("HT-NAV 42C" and "HT-NAV 100C", respectively). A 42% low-endotoxin sucrose (Sigma-Aldrich, St. Louis, USA) solution in DMEM medium (Gibco, Life Technologies; Paisley, UK) was used as control ("matrix") for NAV, per indications of GSK. A vaccine excipient solution was provided by GSK and used as vaccine control ("excipient").

Culture and quantification of TBEV
Live tick-borne encephalitis virus (strain Neudörfl H2J) was obtained from the European Virus Archive (Marseille, France). 300 μL of the virus seed (10^4 TCID$_{50}$/mL) was expanded on Vero E6 cells (ATCC, Rockville, MD) grown in DMEM medium (Gibco) supplemented with 10% fetal calf serum (FCS; Life Science Production, Bedford, UK) and 1% penicillin/streptomycin (Gibco). The virus culture was performed over 21 days, transferring the cell culture supernatant from the inoculum in $0.3*10^6$ cells to $1*10^6$ cells, and then to $2.7*10^6$ cells on days 7 and 14, respectively.

The infectious particles in the supernatant were quantified by plaque assay on A549 cells (ATCC), which are highly susceptible to the virus cytopathic effect (Orlinger et al., 2011). Briefly, monolayers of A549 cells cultured in 12-well culture plates were inoculated with 10-fold dilutions of TBEV-containing cell supernatants for 4 h at 37°C. The cells were overlaid with 2% agarose in 2X MEM medium and incubated for 4 days at 37°C with 5% CO_2. The cells were then fixed with 10% formaldehyde for 1 h, the overlay was discarded, and the cells were stained with crystal violet to visualize the plaques. The virus titers were expressed as plaque-forming units (PFU) per mL.

2.2 Cellular platforms
THP-1 cells
The human monocytic cell line THP-1 (ATCC) was grown in RPMI-1640 medium (Gibco) supplemented with 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate (Gibco) and 50 μM β-mercaptoethanol (Gibco). The cells were used as such or differentiated to a dendritic phenotype (medium supplemented with granulocyte-macrophage colony-stimulating factor and

interleukin-4 (GM-CSF and IL-4; both 1500 U/mL, ProSpec, Rehovot, Israel) added every 2 days over a period of 5 days), or a macrophage phenotype (medium supplemented with 100 nM phorbol 12-myristate 13-acetate (PMA, ThermoFisher Scientific, Waltham, USA) for 48 h, followed by 24 h in PMA-free culture medium). All cells were cultured at a density of $0.3*10^6$ cells/mL in 24-well plates and incubated at 37°C, 5% CO_2.

PBMCs
Buffy coats were purchased from the Dutch blood bank (Sanquin, Groningen, The Netherlands), who had obtained consent from the donors to use the cells for scientific research. PBMCs were isolated as previously described (Tapia-Calle et al., 2017). Briefly, buffy coats were mixed with RPMI-1640 and layered on Ficoll Paque (GE Healthcare, Uppsala, Sweden). After centrifugation, PBMC fractions were collected and red blood cells lysed with ammonium-chloride-potassium (ACK) lysis buffer (ThermoFisher Scientific). PBMCs were then stored in cryopreservation medium (90% FCS, 10% DMSO) in liquid nitrogen until needed. PBMCs were thawed as previously described (Tapia-Calle et al., 2017) and seeded at a density of $2*10^6$ cells/mL in 24-well plates in RPMI-1640 supplemented with 10% FCS, 50 μM β-mercaptoethanol and 1% penicillin/streptomycin. Cells were incubated at 37°C, 5% CO_2.

Monocyte-derived dendritic cells
Monocytes differentiated from thawed PBMCs were isolated using an immunomagnetic negative selection kit, the MagniSort Human pan-Monocyte Enrichment Kit (ThermoFisher Scientific). To obtain immature dendritic cells, monocytes were seeded at a density of $1*10^6$ cells/mL in 24-well plates and cultured in medium supplemented with GM-CSF (450 U/mL) and IL-4 (500 U/mL). Fresh cytokines were added every 2 days over a period of 6 days.

2.3 Cell stimulation
Cells were stimulated for 24 or 48 h with the vaccine formulations (or their control solutions) at dilutions varying from 1:16 to 1:4000 (equivalent to concentrations from 4 to 0.015 μg/mL for NAV and from 0.192 to 0.00075 μg/mL for the adjuvanted vaccine). Incubation with live TBEV was performed for 24 or 48 h at a multiplicity of infection (MOI) of 1, 5 or 10. The TLR7 stimulant resiquimod (R848; 10 μg/mL; Invivogen, Toulouse, France) and human IFNα2a (1000 U/mL; ProSpec) were used as positive controls.

2.4 Toxicity determination, surface marker staining and flow cytometry analysis
To determine the effect of the treatments on cell viability, PBMCs and THP-1 cells were stained with Viability Fixable Dye (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the cells were washed with PBS and resuspended in cold washing buffer (PBS supplemented with 2% FCS and 1 mM EDTA), then incubated for 15 min at room temperature with the dye. Cells were then washed, fixed in 200 μL of 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in PBS for 30 min, washed again,

and finally resuspended in 200 μL of washing buffer for flow cytometry analysis.

In order to examine the expression levels of selected surface markers in THP-1 cells by flow cytometry analysis, APC-labeled recombinant anti-human antibodies (CD11c, CD14, CD32, CD299 and CD120c, all from Miltenyi Biotec) were used for staining according to the manufacturer's instructions. All flow cytometry analyses were performed on a FACSVerse flow cytometer (BD Pharmingen, San Diego, CA, USA). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

2.5 Cell lysis, RNA isolation and RT-qPCR
To detect changes in the gene expression of stimulated cells, cell lysates of different cell subsets were collected, and the mRNA levels of selected genes were analyzed by RT-qPCR.

Cell lysates
Non-adherent cells in culture supernatants were collected in an Eppendorf tube, centrifuged (300 g, 10 min) and lysed by adding 350 μL RLT buffer (Qiagen, Hilden, Germany) with 1% β-mercaptoethanol. The adherent cells were lysed in-well. These lysates were then combined and stored at -20°C until further analysis. For what concerns the evaluation of gene expression in adherent vs non-adherent PBMC subsets, the lysates were instead kept and analyzed separately.

RNA isolation and RT-qPCR
RNA isolation was performed using the RNeasy Mini Kit (Qiagen) following the instructions of the manufacturer. cDNA from the isolated RNA was generated using the PrimeScript RT Reagent Kit (Takara, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. The cDNA was then analyzed by qPCR: the reaction (10 μL 2x ABsolute qPCR SYBR Green Mix (ThermoFisher Scientific), 1 μL 10 mM forward primer, 1 μL 10 mM reverse primer, 1.5 μL cDNA and 6.5 μL H_2O) was carried out for 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C in a CFX96 Touch Real-Time PCR Detection System (Biorad, Hercules, CA). The gene expression levels of the target genes were normalized against the housekeeping gene GAPDH and quantified relative to the expression levels in non-treated cell cultures (primer sequences shown in Tab. S1[1]). Data were analyzed according to the comparative Ct method (Schmittgen and Livak, 2008) and are expressed as fold change.

2.6 Statistical analysis
Significant differences between the responses to the vaccine formulations and to their respective negative controls were determined using the unpaired Student's t-test. Significant differences across multiple groups were determined using two-way ANOVA, applying correction for multiple testing. A p-value < 0.05 was considered significant and is indicated by *; ** indicates $p < 0.01$, and *** indicates $p < 0.001$. Statistical analyses were performed with GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

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3 Results

3.1 THP-1 cells do not show specific responses to TBE vaccine formulations

To develop an *in vitro* system suitable for assessing the conformity of batches in the context of commercial TBE vaccine production, we initially turned to THP-1 cells, a human monocytic cell line extensively used to study monocyte or macrophage functions (Chanput et al., 2014).

We first determined the viability of THP-1 cells upon stimulation with TBE vaccine and non-adjuvanted vaccine (NAV), along with their respective negative controls, i.e., excipient and matrix (Fig. S1[1]). Cells incubated for 24 h with equal dilutions of vaccine or NAV and their controls showed a dose-dependent decrease in viability. The antigen concentrations at which vaccine and NAV were tolerated by the cells differed, likely due to the presence, in the former, of aluminum hydroxide. Even when used at final concentrations as low as 0.2 µg/mL (corresponding to the 1:16 dilution), the vaccine and the excipient induced cell death in > 30% of the cells. In contrast, NAV and matrix were well tolerated, even at 5-10 times higher concentrations.

To assess the activation of THP-1 cells by the treatments, the gene expression levels of *TNF-α* and *IL-12p40* (indicators of an inflammatory response), and of *MxA* and *ISG56* (antiviral genes; transcription of *ISG56* has been shown to increase upon incubation with live TBEV; Overby et al., 2010) were determined. While THP-1 cells responded to the positive controls R848 and IFNα2a, the TBE vaccine and NAV did not induce stronger stimulation than their respective controls (Fig. S2A-D[1]). Responses yielded similar results also after a longer incubation period (Fig. S3[1]). When exposed to live virus, THP-1 cells did, however, show increased expression of *TNF-α*, *ISG56* and *MxA* (Fig. S4[1]). Thus, while the cells do possess the receptor for the entry of TBEV and the machinery necessary for responding to the replicating virus, vaccine and NAV were unable to trigger these responses.

To increase the cells' sensitivity (Starr et al., 2018; Safar et al., 2019; Daigneault et al., 2010) and improve the specificity of the response to the TBE vaccine, THP-1 cells were differentiated to dendritic-like (DC) and macrophage-like (MΦ) cells (Fig. S5[1])[2] (Daigneault et al., 2010; Berges et al., 2005). Yet, the responses remained unspecific: no significant differences between the responses to NAV and matrix were found (Fig. S6A,B[1]), nor between the vaccine and excipient (data not shown). Based on these results, we concluded that, regardless of their differentiation status, THP-1 cells are not suitable for assessing TBE vaccine-induced responses based on the chosen markers.

3.2 Human PBMCs show NAV-specific upregulation of interferon-stimulated genes

As THP-1 cells proved to be an unsuitable platform for assessing TBE vaccine-induced changes in expression of the selected genes, we next turned to primary immune cells. Of note, the TBE vaccination status of the donors in this study was unknown; however, given the absence of a governmental recommendation and the very low incidence of TBE in the Netherlands (with a total of only 12 cases reported so far), it is highly unlikely that the donors had been previously exposed to the virus or the vaccine[3].

The viability of PBMCs, isolated from a healthy donor and cryopreserved, was evaluated after incubation for 24 h with TBE vaccine and NAV. Like the results obtained in THP-1 cells, the tolerability of the vaccine was lower than that of NAV for the same antigen content. The vaccine reduced the cell viability by more than 20% when used at concentrations higher than 0.012 µg/mL (corresponding to a 1:250 dilution), while NAV was well tolerated up to about 1 µg/mL (1:63 dilution in the cell suspension). On average, the primary cells exhibited a slightly higher loss in viability as compared to THP-1 cells undergoing the same treatment (Fig. S7[1]).

We next analyzed the effect of vaccine and NAV on the expression of the previously selected genes. The vaccine increased the expression levels of *IL-12p40*, *TNF-α* and *MxA* considerably (Fig. 1). However, similarly strong effects were observed for the excipient, indicating that the responses were most likely due to the adjuvant rather than to the vaccine. In contrast, NAV did exert responses far exceeding those to the corresponding matrix control, indicated by a NAV-specific increase in expression levels of type I interferon-stimulated genes (ISG) such as *ISG56*, *MxA* (Fig. 1C,D) and *Viperin* (data not shown). In contrast, inflammatory responses were not consistently induced in a NAV-specific manner. Given the high toxicity – at high antigen concentrations – and non-specificity (at low concentrations) of the responses to the adjuvanted vaccine, our experiments hereafter focused on the responses induced by NAV.

To verify the reproducibility of these findings, cryopreserved PBMCs isolated from 10 healthy donors were analyzed for their responses to NAV by RT-qPCR analysis of the candidate biomarkers *ISG56* and *MxA* (Fig. 2A,B). Even though we observed donor-to-donor variability in the degree of cell activation, all donors displayed significantly higher *ISG56* responses to NAV than to the matrix – except for donor #3. Notably, cells from the unresponsive donor also showed absent or limited activation by TLR ligands and IFNα2a used as positive controls (Fig. S8[1]). These results convinced us that, despite the inherent variability in the extent of responses, a platform based on cryopreserved primary PBMCs could be used to evaluate cell activation by the non-adjuvanted vaccine for TBEV.

In order to identify the most responsive subpopulation(s) of PBMCs, we assessed the upregulation of *ISG56* in different subsets of cells obtained from two of the pre-screened donors (donors #1, medium responder, and #7, high responder; Fig. 2C). First, we evaluated the activation of the adherent cells in the PBMC cultures. These cells, which largely consist of monocytes (Van Voorhis et al., 1982), displayed upregulated transcriptional levels of *ISG56* in response to stimulation with NAV, similar to unfractionated PBMC cultures. Non-adherent cells present in the culture supernatant, on the other hand, did not show NAV-specific IFN responses. Differentiating PBMC-derived monocytes into DCs

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[2] https://www.bdbiosciences.com/documents/BD_Multicolor_MonocyteMacrophageDiff_AppNote.pdf

[3] https://www.rivm.nl/tekenencefalitis
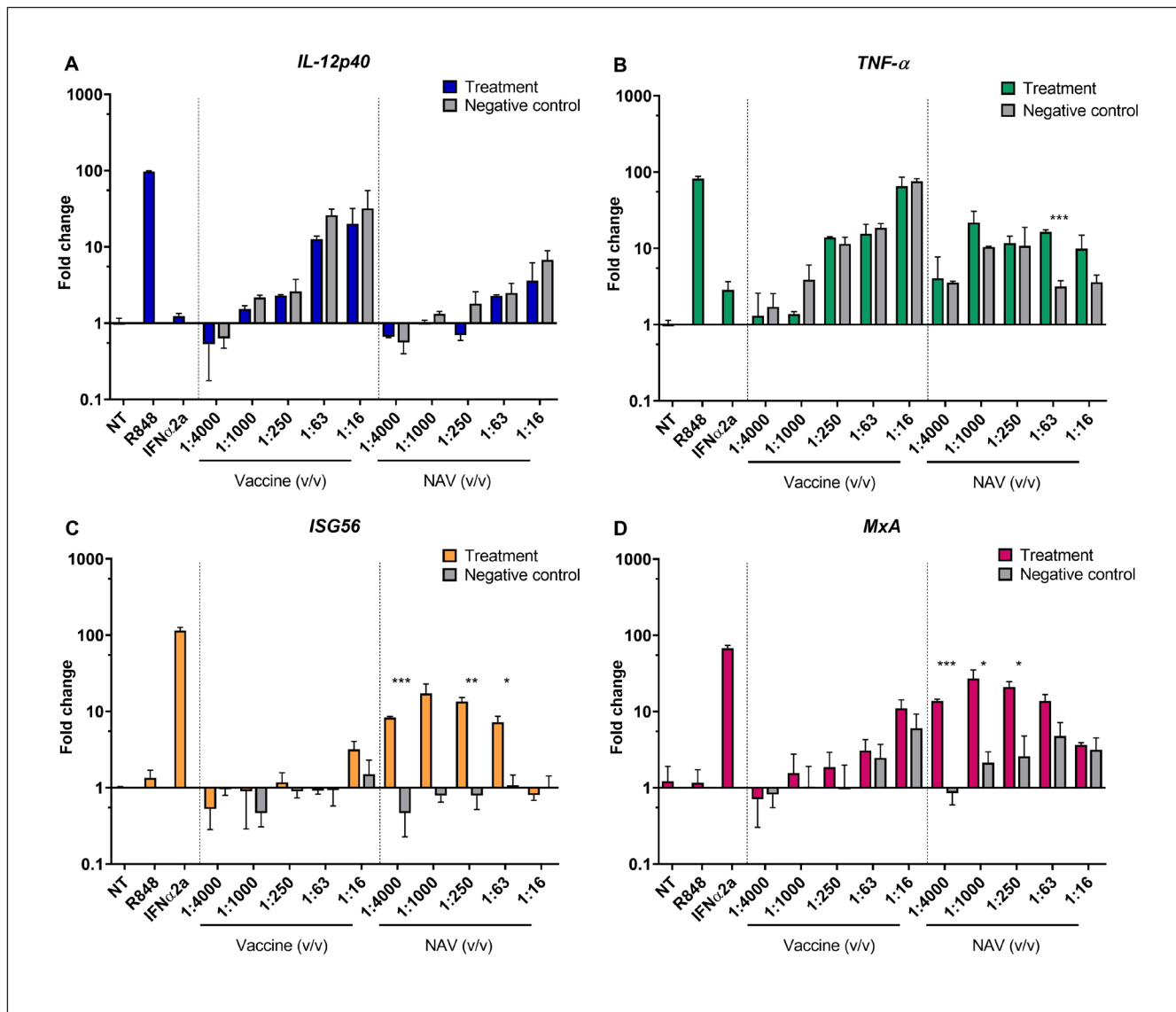
**Fig. 1: Gene expression levels of human PBMCs from a healthy donor exposed to TBE vaccine or NAV**
(A-D) After 24 h of incubation with the indicated stimuli, expression levels of *IL-12p40*, *TNF-α*, *ISG56* and *MxA* were analyzed by RT-qPCR. Concentration of vaccine and NAV is indicated as the dilution factor (v/v) in the medium; it corresponds to 0.75, 3, 12, 48 and 192 ng/mL for the vaccine and to 0.015, 0.06, 0.24, 1 and 4 µg/mL for NAV. Bars represent the mean fold change in gene expression as compared to the non-treated (NT) control ± SD of 3 independent experiments. Levels of significance: *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

resulted in even stronger upregulation of *ISG56* by NAV compared to unfractionated PBMCs or adherent cells. Thus, the capability to respond to NAV resides in the myeloid rather than the lymphoid cell population.

3.3 NAV-specific responses in primary cells are consistent across batches, distinctive of unaltered TBEV antigens, and can be used in a highly sensitive assay
We next aimed to determine whether the identified responses were consistent across different conforming batches of NAV and whether the PBMC platform was sufficiently sensitive in distinguishing high- and low-quality TBE NAV batches. Seven

different batches of NAV induced almost identical levels of upregulation of *ISG56* and *MxA* expression in PBMCs from two different donors, indicating high consistency of the *in vitro* responses to conforming products (Fig. 3A,B). We then artificially produced, from 4 NAV batches, "non-conforming" batches using two different heat treatments, i.e., a 42°C/4-week treatment and a 100°C/15 min treatment, simulating poor NAV handling. These treatments are being investigated for their effect on the binding of TBE vaccine-specific antibodies to the antigens (Akkermans et al., 2020). Exposure of PBMCs to heat-treated non-conforming NAV batches resulted in significantly reduced expression of *ISG56* as compared to exposure to conforming batches. Changes
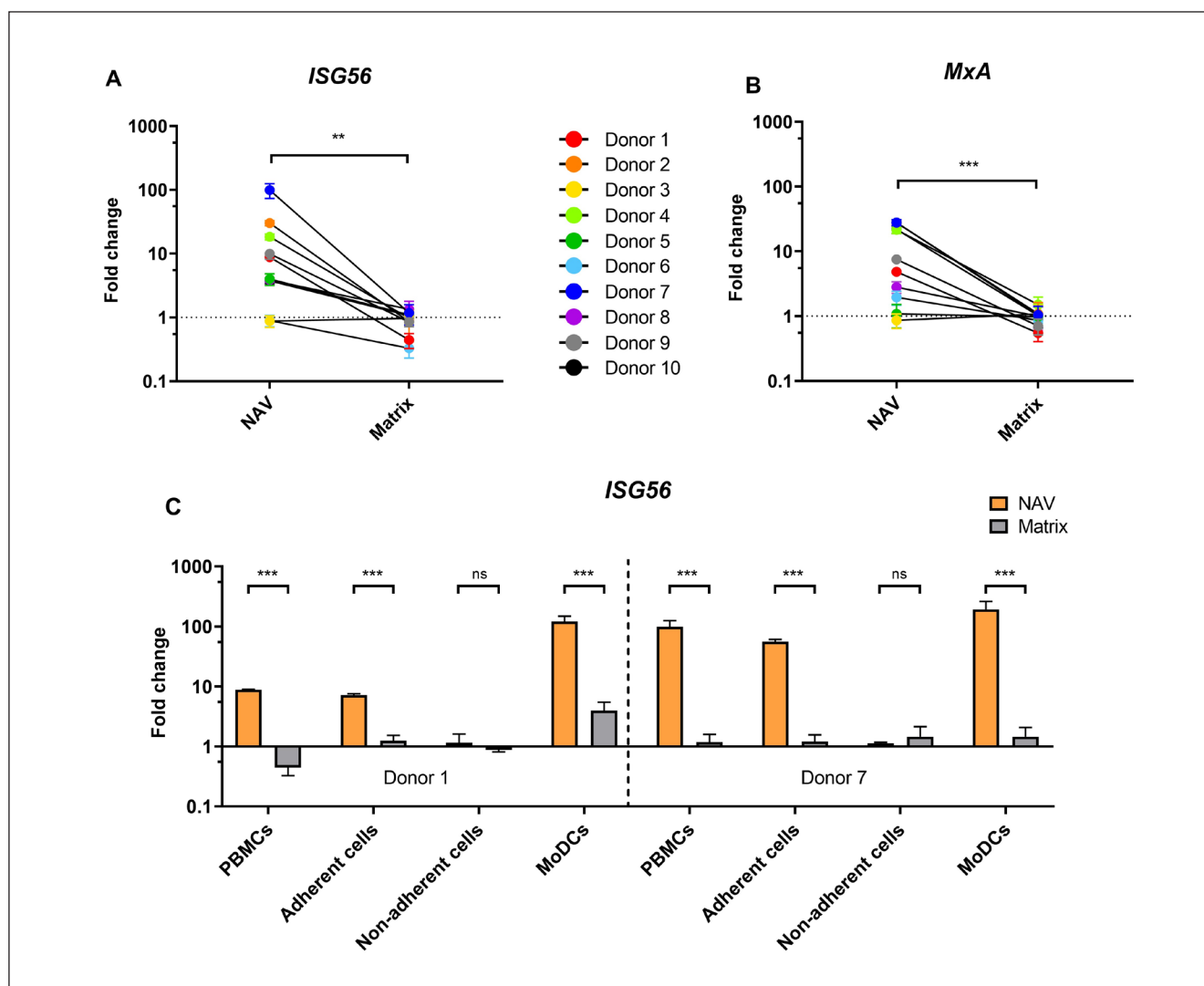
Fig. 2: Responses to non-adjuvanted TBE vaccine in human immune cells from multiple donors
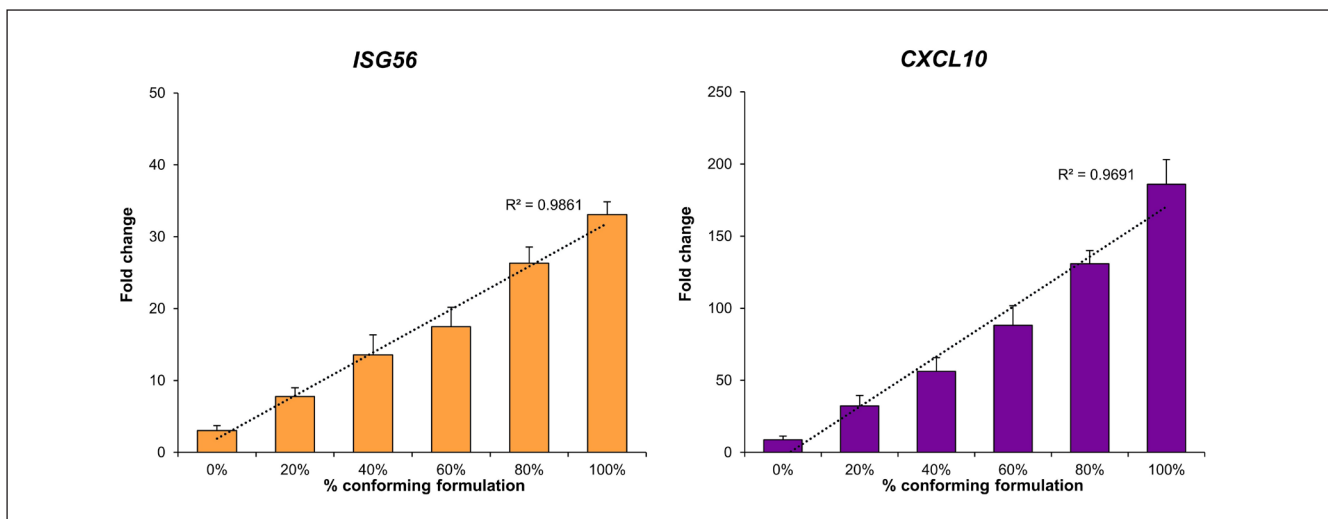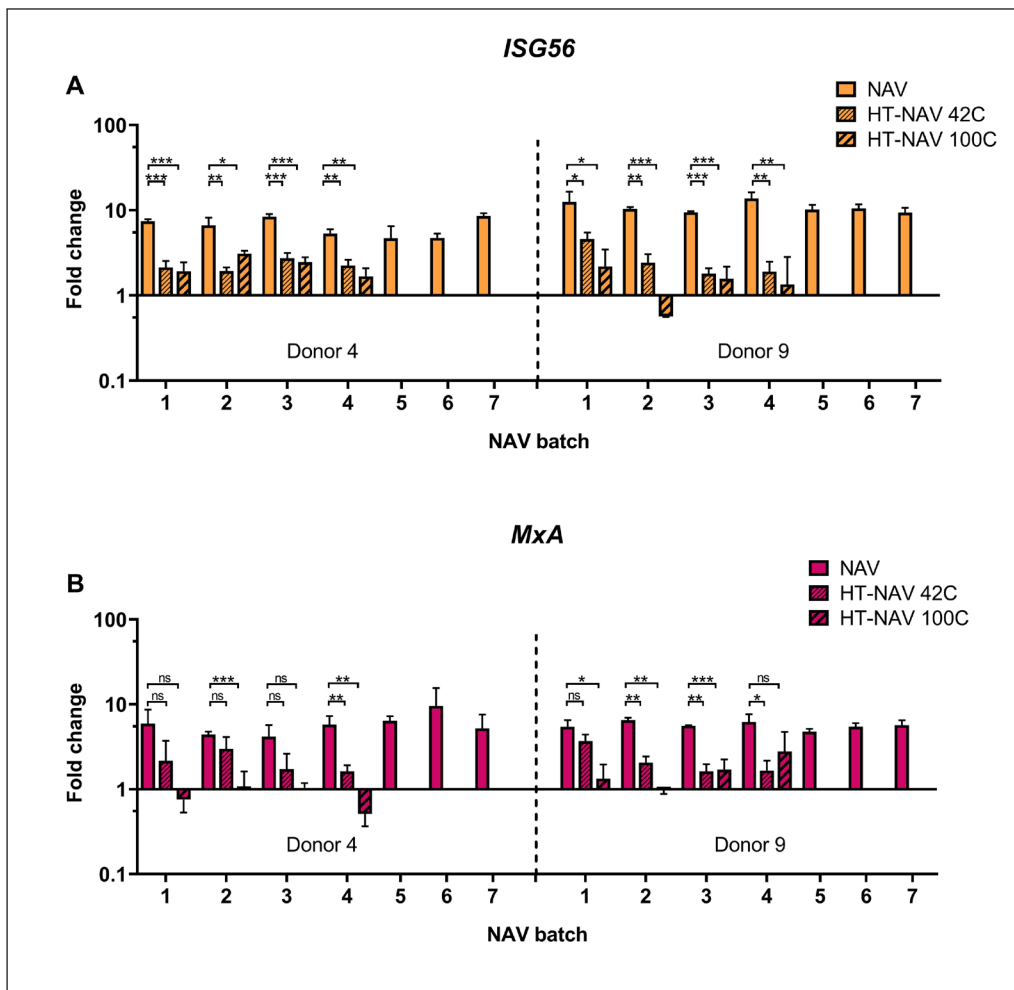(A,B) Expression levels of *ISG56* and *MxA* in 10 donors following NAV (1:250 v/v; 0.24 µg/mL) and matrix stimulation were analyzed by RT-qPCR. (C) Several cell subsets obtained from the PBMCs of donors #1 and #7 were stimulated with NAV (1:250 v/v; 0.24 µg/mL) and matrix, and the expression levels of *ISG56* were analyzed by RT-qPCR. Results represent the mean fold change in gene expression as compared to the non-treated control ± SD of 3 independent experiments. Levels of significance: ns, not significant; *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

in the expression of *MxA* followed a similar trend but were less consistent, with non-significant differences between the responses to some conforming and heat-treated batches.

To evaluate the sensitivity of the PBMC platform in identifying differences between conforming and non-conforming batches, we created mixtures of the two formulations at varying ratios. As a non-conforming formulation for this assay, we used a naturally aged (24 months) batch that elicited poor antiviral responses in PBMCs of a highly responsive donor (#2) to more closely mimic normally occurring non-potent batches. The changes in gene expression of *ISG56* show that, at a very low concentration (0.06 µg/mL, corresponding to a 1:4000 dilution v/v), the NAV-induced responses increase linearly with the percentage of conforming batch present in the mixtures (Fig. 4). Similar results were obtained by

assessing the expression of *CXCL10* (a chemokine found to be upregulated upon stimulation with live TBEV or NAV stimulation; Overby et al., 2010; Etna et al., unpublished results), which was however not evaluated across multiple donors and batches. The responses to mixtures with less than 100% conforming NAV content were all significantly different to those obtained with the optimal formulation (Tab. S2, S3[1]). These promising results, if confirmed across multiple donors, could deliver an assay able to identify losses of active compound as low as 20%.

In conclusion, using the expression of *ISG56* as readout, the PBMC-based *in vitro* system presented here responds in a consistent way to multiple conforming NAV batches and across multiple (responsive) donors, showing significantly reduced responses to non-conforming batches.

Fig. 3: Expression of interferon-stimulated genes in human PBMCs treated with conforming and non-conforming NAV
Changes in gene expression of *ISG56* (A) and *MxA* (B) in donor #4 and #9 following 24 h stimulation with conforming and non-conforming (heat-treated) NAV batches (1:250 v/v; 0.24 µg/mL) were analyzed by RT-qPCR. NAV batches 1-7 were used as conforming batches only; batches 1-4 were also used to generate two sets of heat-treated batches (42°C/4 weeks, 100°C/1 5 min). Bars represent the mean fold change in gene expression as compared to the non-treated control ± SD of 4 independent experiments. Levels of significance: ns, not significant; *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

Fig. 4: Responses in PBMCs treated with mixtures of conforming and non-conforming NAV
(A,B) Changes in gene expression of *ISG56* and *CXCL10* in cells from donor #2 following 24 h stimulation with mixtures of conforming and non-conforming (naturally aged) NAV batches (1:4000 v/v; 0.06 µg/mL) were analyzed by RT-qPCR. % indicates the ratio of conforming to non-conforming batch mixture (e.g., 40% = 40% conforming NAV, 60% non-conforming NAV). Bars represent the mean fold change in gene expression as compared to the non-treated control ± SD of 3 independent experiments.

4 Discussion

In this study, we aimed to identify a cellular platform that could be used for assessing the conformity of TBE vaccine batches *in vitro*. We assessed several cell-based systems for their capacity to discriminate between altered and non-altered TBEV antigens and to display low variability in their responses. Using RT-qPCR analysis, we showed that human PBMCs stimulated with non-adjuvanted TBE vaccine (NAV) displayed changes in the expression of several ISGs, which were: 1) donor-independent in their upregulation, 2) consistent across different batches, and 3) significantly (in the case of *ISG56*) lower in magnitude after stimulation with non-conforming batches. These results support the suitability of a PBMC-based system for the comparative evaluation of TBE NAV batches *in vitro*.

In vitro cell-based systems have been studied extensively for the purpose of vaccine assessment and proposed as a valid replacement method for *in vivo* potency tests (Hoonakker et al., 2015; Nelissen et al., 2009; Hoefnagel et al., 2011; Vandebriel and Hoefnagel, 2012; Tapia-Calle et al., 2017; Leenaars et al., 2001; Ming et al., 2019). Most of these systems use cell lines, such as MUTZ-3 and THP-1, to achieve high reproducibility of the results, a desirable characteristic in a quality control setting. Due to earlier discouraging results with MUTZ-3 cells (Tapia-Calle et al., 2017), we here focused on THP-1 cells as a cell line-based platform for the assessment of vaccine batches. These cells have been used repeatedly as an *in vitro* macrophage model and can also be differentiated to a DC-like phenotype (Higashi et al., 2008; Estrella et al., 2011; Kooijman et al., 2018; Daigneault et al., 2010; Hayman et al., 2017). Yet, despite our efforts in exploring a variety of stimulation and differentiation conditions, we could not identify any TBE vaccine-specific responses in THP-1 cells. The cell line was, however, able to display antiviral responses after incubation with live TBEV. Indeed, previous studies showed that THP-1 cells are permissive to infection with the virus, but mount lower responses to live TBEV than other *in vitro* platforms (Zhang, X. et al., 2016; Wei et al., 2013). Thus, while being a valuable antigen-presenting cell model, THP-1 cells appear to be a weak platform for investigating live or inactivated TBEV.

The inability to exert vaccine-specific responses in THP-1 cells prompted us to turn to PBMCs as a primary cell platform for vaccine screening. We showed that PBMCs, frozen immediately after isolation and preserved in liquid nitrogen for extended times, after thawing readily responded to exposure to NAV by upregulation of *ISG56* and *MxA* expression. In a vaccine screening setting, cryopreserved PBMCs are much more convenient than freshly isolated ones, as they do not present the disadvantages of limited and time-restricted availability or repeated isolation. Contrasting results have been reported regarding the effects of cryopreservation on primary cells' responses, with some studies showing retainment of functionality and population frequency (Anderson et al., 2019; Trück et al., 2014) and others indicating differences in cytokine production and gene expression (Martikainen and Roponen, 2020; Radke et al., 2012). Thus, responses identified in freshly isolated cells should be validated when using cryopreserved PBMCs, with particular attention to the cell subset(s) necessary for the designed assay. In a head-to-head comparison of monocyte-derived DCs dif-

ferentiated from fresh or frozen PBMCs, Tapia-Calle et al. (2017) found that cryopreservation did not affect the cells' ability to respond consistently to vaccine candidates. The retained functionality of monocytes in cryopreserved PBMCs proved to be essential for our assay, as the activation induced by NAV was found to be dependent on the responses of the myeloid cell fraction. Cryopreserved PBMCs are therefore an advantageous and reliable platform for the *in vitro* test described here.

A possible problem of using a primary cell-based platform is inter-individual variability. In our study, we found that while the responses to the non-adjuvanted vaccine differed quantitatively among donors, the changes in expression of ISGs were qualitatively consistent and significant compared to non-treated or matrix-treated cells. The presence of non-responding donors (in our case 1 out of the 10 we screened) can be an issue when assessing vaccine responses *in vitro*. However, non-responders can be identified by the lack of cell activation upon treatment with pattern recognition receptor ligands. For using a PBMC-based assay in an industrial setting – which requires quantitatively consistent results – donor cells could be pre-screened, selected based on their responsiveness to reference compounds (such as LPS and IFN) and to reference vaccine batches, and then cryopreserved. To further mitigate donor-dependent variability, pooling of PBMCs from several donors could be implemented, as it has been shown to reduce inter-assay variation in other *in vitro* assays (Solati et al., 2015; Wieczorek et al., 2013). The data presented shows that, among the biomarkers investigated, *ISG56* performed best with respect to specificity and sensitivity. Further analyses focusing on *CXCL10* or other interferon-stimulated genes (e.g., *ISG15* and *ISG54*, highly upregulated in response to live TBEV, Yang et al., 2020) might reveal other promising markers for this purpose.

Our results demonstrate the ability of TBE NAV batches to consistently induce the transcription of selected interferon-stimulated genes in human primary cells. Indeed, pathway enrichment analysis of microarray data has shown that type I IFN-related genes are common early markers in PBMCs stimulated with several viral vaccines (Zhang, J. et al., 2015). In contrast, selected inflammatory markers were not associated with the response to NAV, which was unexpected as pro-inflammatory cytokines are often upregulated in PBMCs stimulated with whole inactivated vaccines (Stoel et al., 2015; Sasaki et al., 2018). As the molecular mechanisms involved in cell activation by TBEV (live or inactivated) are still poorly understood (Lindqvist et al., 2018), the described PBMC platform could be a valuable tool for the identification of relevant immune pathways.

A limitation of our *in vitro* system is that it could only assess the quality of the non-adjuvanted vaccine and not that of the final vaccine product. The evaluation of the adjuvanted TBE vaccine by the PBMC-based platform proved to be unfeasible due to the low concentration of viral antigens and, more importantly, the high alum content in the adjuvanted formulation. While the adjuvant is safe for human use (Petrovsky, 2015), a considerable amount of evidence indicates that aluminum can interfere with *in vitro* assays and that the toxicity of aluminum hydroxide in cell-based assays can affect the viability of the cellular platform (Mold et al., 2016; Heydenreich et al., 2014; Ko et al., 2017; Minor, 2015). Our results show that the PBMC-based system could not tolerate the adjuvant-

ed formulation at antigen concentrations required for vaccine-specific responses. WHO guidelines indicate that, for nonclinical and initial clinical evaluation of aluminum-adjuvanted vaccines, the potency assessment may require multiple tests, including potency tests prior to adsorption with the adjuvant (WHO, 2014). The assay described here could therefore be used to evaluate the quality of the TBEV antigen in the pre-adsorption product, thereby allowing identification of inferior batches for commercial purposes but also for process performance qualification before they enter expensive and ethically problematic animal testing.

To further reduce the use of animal-derived materials (such as FCS), the literature suggests that, in principle, human primary cells can be cultured and cryopreserved in serum-free conditions (Janetzki et al., 2010; Jeon et al., 2010; Germann et al., 2011); however, an initial readout-specific comparison of the cell responses in FCS-supplemented and FCS-free media should be performed.

The importance of cell-based approaches for the quality control of established vaccines has been emphasized in recent years by various international organizations (Lang et al., 2018; Schutte et al., 2017; Verthelyi et al., 2011). The concerted efforts of many research groups and consortia (e.g., VAC2VAC, a collaborative research project funded by the Innovative Medicine Initiative) has resulted in several studies examining replacement, reduction and refinement (3Rs) principles in the context of vaccine production (Hoonakker et al., 2015; Leenaars et al., 2001; Coombes et al., 2012). Indeed, there is growing scientific evidence that 3Rs strategies are applicable in vaccine development, evaluation and release. The consistency approach facilitates this transition, as it is based on a lot testing strategy that requires animal tests only for the profile definition of the reference product. Then, verification of the conformity between the newly produced and the reference batches can be performed through several *in vitro* assays (Hendriksen, 2009). In this context, our assay could be included in a panel of *in vitro* analyses (Akkermans et al., 2020) that can eventually replace animal testing for batch release control.

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Conflict of interest
AS, MPE, EMC and AH report no conflict of interest.

Author contributions
AS, MPE, EMC, and AH were involved in the conception and design of the study. AS acquired the data. AS, MPE, EMC and AH analyzed and assessed the results. AS, MPE, EMC and AH were involved in methods selection. All authors were involved in drafting the manuscript or critically revising it for important intellectual content. All authors had full access to the data and approved the final manuscript.

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